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Simple and Fast Lateral Flow Test for Classification of Leprosy Patients and Identification of Contacts with High Risk of Developing Leprosy

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The interruption of leprosy transmission is one of the main challenges for leprosy control programs since no consistent evidence exists that transmission has been reduced after the introduction of multidrug therapy. Sources of infection are primarily people with high loads of bacteria with or without clinical signs of leprosy. The availability of a simple test system for the detection of antibodies to phenolic glycolipid-I (PGL-I) of *Mycobacterium leprae* to identify these individuals may be important in the prevention of transmission. We have developed a lateral flow assay, the ML Flow test, for the detection of antibodies to PGL-I which takes only 10 min to perform. An agreement of 91% was observed between enzyme-linked immunosorbent assay and our test; the agreement beyond chance (kappa value) was 0.77. We evaluated the use of whole blood by comparing 539 blood and serum samples from an area of high endemicity. The observed agreement was 85.9% (kappa = 0.70). Storage of the lateral flow test and the running buffer at 28°C for up to 1 year did not influence the results of the assay. The sensitivity of the ML Flow test in correctly classifying MB patients was 97.4%. The specificity of the ML Flow test, based on the results of the control group, was 90.2%. The ML Flow test is a fast and easy-to-perform method for the detection of immunoglobulin M antibodies to PGL-I of *M. leprae*. It does not require any special equipment, and the highly stable reagents make the test robust and suitable for use in tropical countries.

Leprosy, a disease caused by *Mycobacterium leprae*, particularly affects the less privileged parts of the population in countries where the disease is endemic. This intracellular bacillus is assumed not to be very pathogenic, most infections do not result in chronic disease but in skin lesions that heal spontaneously (13). Present forecasts suggest that, despite the slow decline in leprosy transmission, millions of individuals will continue to be infected and develop disease in the next 20 years, notwithstanding the intense efforts to eliminate leprosy as a public health problem. (A. Meima, W. C. Smith, G. J. van Oortmarssen, J. H. Richardus, and J. D. F. Habbema, submitted for publication). Leprosy can be successfully treated with multidrug therapy. Delayed diagnosis increases the chance that leprosy is spread in the community and results in more-severe nerve damage. Interruption of leprosy transmission is one of the main challenges for leprosy control programs. No consistent evidence exists that the incidence of leprosy has been significantly reduced after the introduction of multidrug therapy (20). Sources of infection are particularly patients with high loads of bacteria and, possibly, infected persons in which the clinical signs have not yet become apparent.

The presence of antibodies to the *M. leprae*-specific phenolic glycolipid-I (PGL-I) correlates with the bacterial load of a leprosy patient (17). The large majority of paucibacillary (PB) patients are seronegative, whereas the large majority of multibacillary (MB) patients are seropositive (1, 5, 6, 8, 9). It has been shown that the presence of PGL-I antibodies can be used to classify confirmed leprosy patients as MB or PB for treatment purposes (3, 4). In addition, it was shown that PGL-I-seropositive contacts of leprosy patients have a higher risk of developing leprosy compared with PGL-I-seronegative contacts and that when they develop the disease, it is primarily MB (12). Consequently, identification of antibodies to PGL-I in contacts of leprosy patients may lead to earlier detection of disease and ultimately to prevention of transmission. A simple assay is required to routinely screen the contacts of leprosy patients.

Here, we describe a newly developed simple and rapid immunochromatographic flow test, the ML Flow test, for the detection of immunoglobulin M (IgM) to PGL-I in 10 min. In this study, we have investigated the performance of the ML Flow test for use on serum and whole-blood samples.

MATERIALS AND METHODS

ML Flow test. The ML Flow test is composed of a nitrocellulose detection strip that is flanked at one end by a reagent pad made from fiber-fleece containing the dried colloidal gold-labeled anti-human IgM antibody and at the other end by an absorption pad. A sample application pad flanks the reagent pad in turn (Fig. 1). The semisynthetic 3,6-di-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3-di-*O*-methyl-α-L-rhamnopyranosyl-(1→2)-3-*O*-methyl-α-L-rhamnopyranose linked to bovine serum albumin (NT-P-BSA) (14) was used as the antigen. The trisaccharide represents the unique sugar moiety of the *M. leprae* PGL-I. The NT-P-BSA was deposited as a 1-mm-wide line onto the nitrocellulose strip. Human IgM was deposited as a second line parallel to the test line to function as a reagent control.

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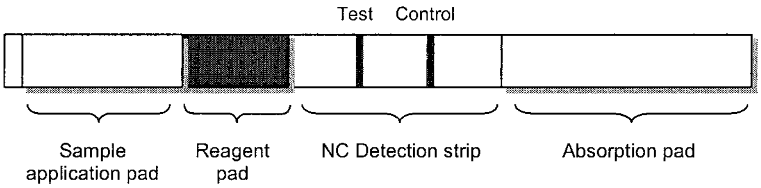

FIG. 1. Diagram of the ML flow test. NC, nitrocellulose.

The composite was backed by a support and cut into 5-mm-wide test strips to fit into a plastic housing with a round sample application well positioned above the sample pad and a square detection window positioned above the detection strip.

The amounts of antigen and detection reagent were optimized in a step-by-step procedure with a panel of positive and negative control sera. The assay is performed by the addition of 5 μl of undiluted serum or whole blood to the sample well followed by the addition of 130 μl of running buffer (phosphate-buffered saline containing 0.66 mg of BSA/ml and 3% Tween 20). The test was read after 10 min for serum and after 5 and 10 min for blood. The test result was only considered valid when the control line was clearly visible. The test is scored positive when a distinct staining of the test line is observed (Fig. 2, lanes 1+ to 4+). When no staining (Fig. 2, lane −) or faint staining (Fig. 2, lane +/−) is observed, the result is considered negative. To increase stability, devices are individually packed in a moisture-resistant sachet.

Study groups. Test performance was determined on the following samples. (i) Five hundred sixty-one serum samples collected in 3 areas of high leprosy endemicity (Manaus in Brazil, South Sulawesi in Indonesia, and Cebu in The Philippines) and 20 samples from an area of low endemicity (Ghana). The sera were derived from the following groups: (a) 114 newly diagnosed MB patients, (b) 85 newly diagnosed PB patients, (c) 42 household contacts of leprosy patients, (d) 106 patients with skin diseases other than leprosy (including 20 from patients with Buruli ulcers from Ghana), (e) 234 healthy individuals (control group). (ii) Ninety-nine serum samples came from an area of nonendemicity (The Netherlands) (control group). (iii) Fifty-nine serum samples were obtained in The Netherlands from patients with various diseases other than leprosy (control group), including patients with tuberculosis ($n = 12$), human immunodeficiency virus ($n = 6$), hepatitis A ($n = 3$), hepatitis B ($n = 6$), syphilis ($n = 6$), malaria ($n = 9$), toxoplasmosis ($n = 6$), and autoimmune disease ($n = 5$) and rheumatoid factor-positive patients ($n = 6$).

Both the ML Flow test and IgM enzyme-linked immunosorbent assay (ELISA) carried out according to Bührer et al. (4) were performed on these samples.

The ML Flow test performance with whole-blood samples was evaluated in a primary health center setting in an area of leprosy endemicity (Curionópolis, Pará, Brazil). Heparinized blood and serum samples were collected from 539 individuals (including newly diagnosed and treated leprosy patients, contacts, and healthy endemic controls) and tested immediately. All patients gave informed consent for serological testing; samples were coded and could not be related to patient names.

Storage experiments. The ML Flow test and the detection reagent were stored for 1 year at three different temperatures (4, 28, and 45°C) and for 2 months at 55°C. The test strips' performance was checked by using a panel of 14 serum samples at various time points.

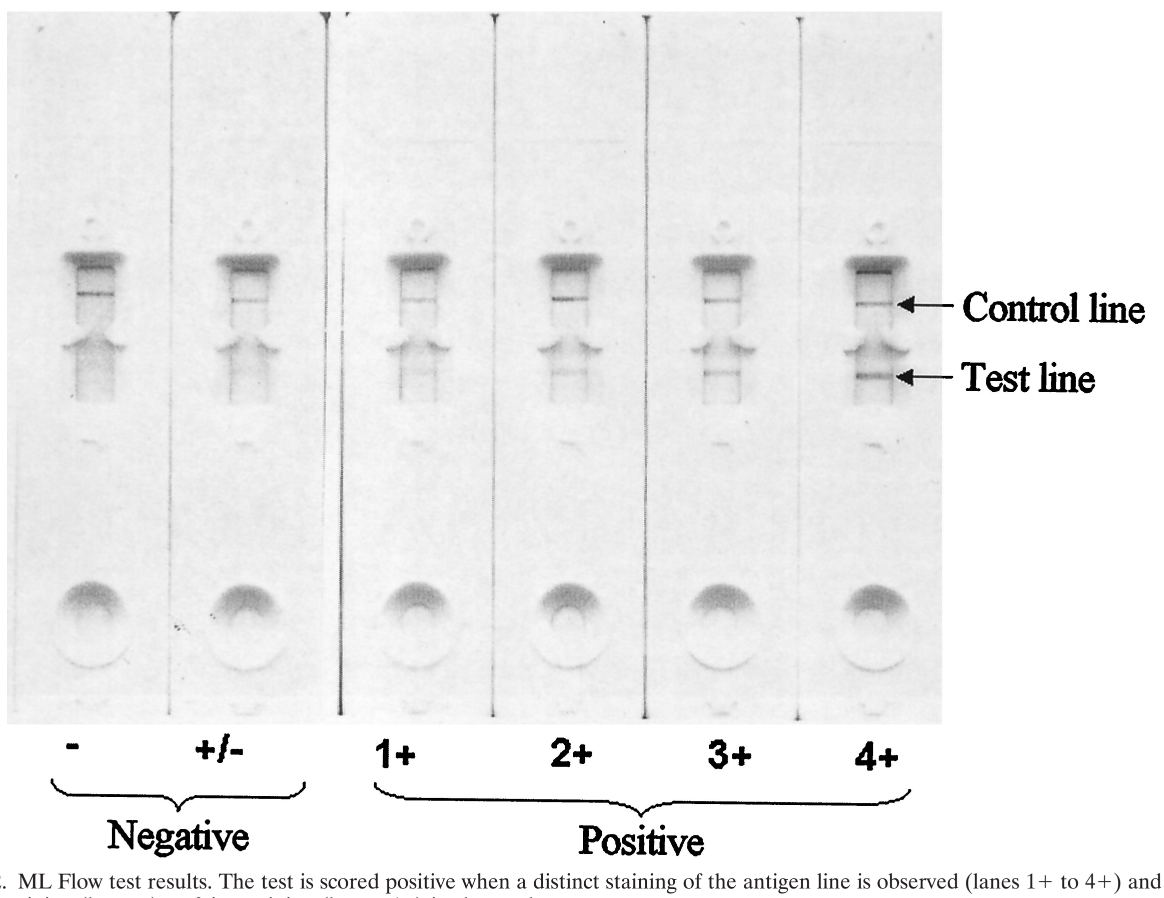

FIG. 2. ML Flow test results. The test is scored positive when a distinct staining of the antigen line is observed (lanes 1+ to 4+) and negative when no staining (lane −) or faint staining (lane +/−) is observed.

TABLE 1. Comparison between ELISA and ML Flow test on 739 serum samples

ML Flow test result	No. of samples with ELISA result		Total no. of samples
	Positive	Negative	
Positive	161	45	206
Negative	21	512	533
Total	182	557	739

Statistical evaluation. Data were analyzed by using Epi-info, version 6. The concordance between the test results of the two assays for a group of sera was determined by calculating the observed agreement and kappa values (κ). Generally, a κ value of 0.60 to 0.80 represents a substantial agreement beyond chance, and a κ value of >0.80 represents almost perfect agreement beyond chance.

RESULTS

Comparison between ELISA and ML Flow test. Table 1 shows the comparison between the ML Flow test and ELISA for 739 sera from the Royal Tropical Institute serum bank. A concordant result was observed for 673 samples in total. The observed agreement between ML Flow and ELISA results was 91% (κ = 0.77; 95% confidence interval [CI], 0.70 to 0.84).

Seropositivity according to the classification of the study group. The ML Flow test gave a positive result in 97.4% of the MB patients, 40% of the PB patients, 28.6% of the household contacts, and 9.8% of the controls (Table 2). Of the 98 MB patients with a bacterial index of at least 2, 97.8% were ML Flow test positive. The sensitivity of the ML Flow test to correctly classify MB patients was 97.4% (95% CI, 93 to 99). The specificity of the ML Flow test based on the results of the total control group was 90.2% (95% CI, 87 to 93) or 86.2% (95% CI, 82 to 90), if individuals from areas of nonendemicity are excluded. When testing samples from people with skin diseases other than leprosy, there was no particular skin disease that could be associated with (high) seropositivity.

Comparison between the ML Flow test result when using whole blood and serum. When testing 539 paired serum and whole-blood samples in the ML Flow test, a concordant result was observed for 463 samples. The observed agreement was 85.9% (κ = 0.70; 95% CI, 0.62 to 0.79) (Table 3).

ML Flow test results with whole blood. When testing 238 whole-blood samples with the ML Flow test with 5 and 10 μl of whole blood, a concordant result was observed for 210 samples (Table 4). The observed agreement between the test performed with 5 and 10 μl of whole blood was 88.2% (κ = 0.76; 95% CI, 0.63 to 0.89).

In all experiments performed with sera, the test results were read after 10 min. When whole blood was used, the results were read after both 5 and 10 min. No difference in the result was observed, but the use of whole blood caused some staining in the nitrocellulose paper which was less noticeable after 5 min than after 10 min.

Reproducibility. A second observer read the results of 739 ML Flow tests on serum samples, and the results were compared with the results of the first reader. When reading results as positive or negative, the agreement was 96% (κ = 0.90; 95% CI, 0.82 to 0.97). When reading the test results as negative, plus/minus, 1+, 2+, 3+, or 4+, 83% (616 results) were in agreement (κ = 0.73; 95% CI, 0.69 to 0.77) and the remaining 17% (123 results) were read one step higher or lower than by the other reader.

Two other observers independently read the results of 539 ML Flow tests, and their results were compared with those of the first reader. The agreements were 94.4% for observer 1 (κ = 0.82; 95% CI, 0.73 to 0.90) and 96.8% for observer 2 (κ = 0.9; 95% CI, 0.81 to 0.98). In addition, the results were also read as negative, plus/minus, 1+, 2+, 3+, or 4+. Of 539 readings, 84.2% (κ = 0.76; 95% CI, 0.70 to 0.81) and 79.2% (κ = 0.66; 95% CI, 0.60 to 0.71) were in agreement. All the discordant readings were read only one step higher or lower than by our original reader.

Storage. There was no change of activity of the ML Flow test strips when stored up to 1 year at 4 to 45°C. Storage of ML Flow test strips for 2 months tested at 55°C results in no

TABLE 2. ML Flow test positivity according to the classification of the study group

Study group classification	Total	No. of positives (%, 95% CI)
MB patients	114	111 (97.4, 93–99)
PB patients	85	34 (40.0, 30–51)
Household contacts	42	12 (28.6, 16–45)
Controls		
From areas of endemicity		
Healthy individuals	234	28 (12.0, 10–18)
Other skin diseases	106	19 (17.9, 11–27)
From areas of nonendemicity		
Other diseases	59	2 (3.4, 0–12)
Healthy individuals	99	0 (0, 0–4)
Total controls	498	49 (9.8, 7–13)

TABLE 3. Comparison between ML flow test on serum samples and on blood samples, both performed in a primary health care center setting

Result for blood sample	No. of serum samples with result		Total no. of samples
	Positive	Negative	
Positive	172	24	196
Negative	52	291	343
Total	224	315	539

TABLE 4. Comparison of ML flow test results with 5 and 10 μl of blood

Result with 5 μl of blood	No. of 10-μl blood samples with result		Total no. of samples
	Positive	Negative	
Positive	86	11	97
Negative	17	124	141
Total	103	135	238

detectable loss of activity. The detection reagent was stable for at least 1 year at 28°C or 3 months at 45°C.

DISCUSSION

The availability of semisynthetic PGL-I derivatives (2, 10, 14, 15) has enabled the development and use of serological tests for the detection of leprosy-specific antibodies. These assays include ELISA, the gelatin particle agglutination test, the ML Dipstick, and now the immunochromatographic strip test (ML Flow test). The ML Flow test is the most rapid and easily applicable assay.

We showed that the ML Flow test gave a good correlation with the ELISA results (91%; $\kappa = 0.77$), that it can be used on finger prick blood and serum alike ($\kappa = 0.70$), giving reproducible results in 5 to 10 min, and that the ML Flow test can be kept outside the refrigerator and is stable for at least 1 year at 45°C. The amount (5 to 10 μl) of whole blood used is not critical, meaning that heparin-coated capillary tubes can be used for blood collection and direct application.

As the ML Flow test is scored by visual inspection for staining of the antigen line, reading of the test is therefore subjective. Faint staining in the ML Flow test must be considered negative since the aim of the test is to detect people with a relatively high bacterial load. Still, the agreement observed between readers in the laboratory and in the field settings was good (96% and 94%, respectively) with κ values above 0.8, representing an almost perfect agreement beyond chance.

All these factors make the assay very suitable for use at different levels of the health care system, including the primary health center.

Classification of leprosy patients for treatment purposes is mostly based on counting the number of lesions (less than 6 skin lesions, PB; 6 or more lesions, MB) (21), but this method is unsatisfactory and subject to error (11). In confirmed leprosy patients, high specific antibody levels in general signify a high bacterial index and the absence of specific antibodies signifies a negative bacterial index (16). Thus, after diagnosis of a leprosy patient, the antibody response to PGL-I can be used for the classification as MB or PB for treatment purposes (4).

The ML Flow test, like all other serological tests for leprosy, is not a diagnostic tool, as the majority of PB patients do not develop detectable levels of antibodies, but it can be used as a tool for classification after the initial diagnosis has been made based on clinical signs and symptoms. Correctly classifying leprosy patients will (i) make leprosy control more cost efficient by preventing overtreatment and (ii) prevent transmission by avoiding undertreatment of MB patients that could otherwise be a source of infection due to relapse (3). In our study population, the sensitivity of the ML Flow test to correctly classify MB patients was 97.4% (95% CI, 93 to 99). In the group of untreated PB patients studied, the seropositivity was 40% with the ML Flow test, which is rather high; the seropositivity in PB patients has usually been reported to be in the range of 15 to 30% (1, 7, 8, 18). Using ELISA as our "gold standard," we found the seropositivity in this particular group of samples to be 38%, which is not significantly different from the result of the ML Flow test.

The specificity of the ML Flow test based on the results of the total control group was 90.2% (95% CI, 87 to 93) or 86.2% (95% CI, 82 to 90) if individuals from an area of nonendemicity are excluded. The ML Flow test gave a positive result in 9.8% of 498 control sera and in 1.3% of the 158 controls from an area of nonendemicity, which was similar to results obtained with a parallel ELISA study (results not shown). This latter observation confirms the specificity of the ML Flow test, even when the percentage of positive results in the control groups from an area of endemicity (consisting of people who may have been in contact with the leprosy bacillus) was relatively high. We did not see higher seropositivity in the tuberculosis and Buruli ulcer patient groups, indicating that there is no cross-reactivity with the glycolipids from the mycobacteria responsible for these infections.

PGL-I-based serological tests cannot be used as screening tools in the general population since not every person that is exposed and develops antibodies to *M. leprae* will ultimately develop clinical disease (19). The ML Flow test is not proposed for the screening of the whole population in communities where leprosy is endemic. It was previously shown with ELISA that seropositive contacts of leprosy patients have a relative hazard of 8 to develop leprosy and 56.1 to develop MB leprosy compared to seronegative contacts (based on reference 12). The results clearly show that serology can be used as a tool for the identification of contacts of leprosy patients with a high risk of developing leprosy. Screening contacts of leprosy patients in order to find and follow up or treat those at increased risk of developing leprosy may ultimately prevent transmission.

In conclusion, the ML Flow test was developed as a simple, stable, and rapid tool for two applications: (i) for the correct classification of newly diagnosed leprosy patients and (ii) to identify those contacts of leprosy patients that have an increased risk of developing leprosy in future.

In order for the leprosy control programs to be successful, it is essential both to treat patients accurately and to ensure that transmission of this slow chronic disease is prevented. The ML Flow test could contribute to these aims.

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